

Larval *Crassostrea* bivalve and *Artemia* brine shrimp bioassays to assess toxicity and micropredation by the heterotrophic dinoflagellates *Cryptoperidiniopsis brodyi* and *Pfiesteria piscicida* from Australian waters

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The capability of the heterotrophic Australian marine dinoflagellates Cryptoperidiniopsis brodyi and Pfiesteria piscicida to impact on larval Pacific oyster (Crassostrea gigas) and brine shrimp (Artemia salina) nauplii was investigated. An attractant response of the heterotrophs toward actively swarming Artemia was not observed and no mortality occurred. In contrast, the dinoflagellates became active and exhibited attacking behavior toward planktonic oyster larvae (below 1 mm size) within a few seconds. The oyster larvae survived 2500 cells mL⁻¹ for 2 days, but mortality increased to 82–88% by day 12. Aqueous dinoflagellate cell extracts collected from the bioassay that induced oyster kills were tested in a further larval bivalve bioassay but no mortality was observed. Oysters over 2 mm in size survived the physical attack by the heterotrophs and remained alive during the bioassay period. Changes in zoospore and cyst abundances in the presence of oyster larvae were also documented. More actively swarming zoospores and 2.5-fold higher cell numbers were produced in the presence of oysters compared to control cultures. These results indicate that mortalities of the planktonic larvae can be induced by micro-predatory feeding behavior of C. brodyi and P. piscicida.

INTRODUCTION

The heterotrophic dinoflagellate *Pfiesteria piscicida* Steidinger et Burkholder was first discovered in 1988 (Smith *et al.*, 1988) and has been proposed as a causative agent of fish kills (Burkholder *et al.*, 2001a). *Cryptoperidiniopsis brodyi* Steidinger et Litaker known as a *Pfiesteria*-like dinoflagellate is another heterotroph which often co-occurs with *Pfiesteria* species at fish-kill sites in estuaries (Marshall *et al.*, 1999; Steidinger *et al.*, 2006). These algae have been known to share similar life cycle stages and feeding behaviors (Litaker *et al.*, 2002; Parrow and Burkholder, 2004). *Cryptoperidiniopsis brodyi* is

morphologically indistinguishable from *Pfiesteria* species by light microscopy (LM) but differs genetically and in the fine structure of plate tabulation (Steidinger *et al.*, 1996, 2006; Litaker *et al.*, 1999). These dinoflagellates possess a peduncle (feeding tube) that can be extended between its sulcal plates and is used to feed on micro-algae, bacteria, fish and other organisms (Burkholder and Glasgow, 1995; Burkholder *et al.*, 2001a). This feeding behavior known as myzocytosis (Litaker *et al.*, 2002; Vogelbein *et al.*, 2002) can cause tissue damage and contribute to the death of fish (Burkholder *et al.*, 2001c; Vogelbein *et al.*, 2002).

Pfiesteria piscicida has been claimed to have the ability to produce potent ichthyotoxins in the presence of fish or fresh fish excreta (Burkholder and Glasgow, 1995; Marshall *et al.*, 2000; Burkholder *et al.*, 2001b, 2005). Fish excreta and secretions were suggested to stimulate a strong chemosensory response by *Pfiesteria* species and induce “ambush-predator” behavior toward fish prey (Burkholder and Glasgow, 1995; Cancellieri *et al.*, 2001). Early studies claimed that there exist several functional types in this alga based on its capability of toxin production (Burkholder and Glasgow, 1995; Burkholder *et al.*, 2001b; Cancellieri *et al.*, 2001). TOX-A functional type is strongly attracted to fish prey and actively toxic (fish killing mode), TOX-B functional type is temporarily non-toxic when removed from access to live fish or other prey, and NON-IND type called benign or non-inducible cannot produce bioactive substances that cause fish death (Burkholder *et al.*, 2001b). An early source of a claimed toxic fraction was disproved to be a contaminant from artificial seawater medium, which can degrade water quality and contribute to fish mortality (Berry *et al.*, 2002; Vogelbein *et al.*, 2002; Lovko *et al.*, 2003). More recently, Moeller *et al.* (2007) proposed metal-mediated free radical production as a potential mechanism of ichthyotoxicity by *Pfiesteria*. In other work, fish kills were demonstrated to occur only upon direct physical contact with *Pfiesteria* species (Vogelbein *et al.*, 2002).

Survival and physiology of bivalve larvae also can be significantly affected by the toxins of dinoflagellates because early developmental stages are more vulnerable than adult stages of bivalves (Shumway and Cucci, 1987; Gainey and Shumway, 1988; Shumway, 1990). Few studies have been conducted to understand interactions between bivalves and *Pfiesteria* species (Burkholder and Glasgow, 1997a; Springer *et al.*, 2002; Shumway *et al.*, 2006). An effect of the alleged toxicity of *Pfiesteria* species on bay scallop larvae was documented, with 100% larval mortality reported in 1 h (Springer *et al.*, 2002).

Microscale bioassays have been used extensively in aquatic toxicology because of advantages such as simplicity, rapidity and cost-effectiveness (Burton and Fisher, 1990; Blaise *et al.*, 1998; Nacci *et al.*, 1998; Weis and Weis, 1998). Microscale bioassays allow for higher numbers of replicates and accurate statistical analyses (Hunt *et al.*, 1998; Lovko *et al.*, 2003). *Pfiesteria* species have also been used in small-volume bioassay formats, with comparable results to larger volume assays (Burkholder *et al.*, 2001a; Berry *et al.*, 2002; Springer *et al.*, 2002; Vogelbein *et al.*, 2002; Lovko *et al.*, 2003). Small-scale bioassays allow for detailed observations of the entire system under the light microscope with minimal growth of micro-contaminants due to shorter assay time and small-scale (Lovko *et al.*, 2003).

Cryptoperidiniopsis brodyi appears to be widely distributed in Australia and *P. piscicida* was also found in ballast water from Indonesia (Park *et al.*, 2007a). The objective of this study was to examine capability of *P. piscicida* from Indonesia and *C. brodyi* from Australia to cause Pacific oyster *Crassostrea gigas* (Thunberg, 1793) and brine shrimp *Artemia salina* (Linnaeus, 1758) deaths. The Pacific oyster and brine shrimp were chosen for bioassays because *C. gigas* is an important commercial oyster in Australia and *A. salina* is a widely used standard organism in toxicity tests. Additionally, changes in cell numbers of *C. brodyi* were documented during larval bivalve bioassays.

METHOD

Pfiesteria piscicida and *C. brodyi* cultures

The following strains were used in this study: *C. brodyi* strains CBWA12 and CBSA4 from West and South Australia respectively, *C. brodyi* strains CBDE10, CBHU1 and CBDE1 from Tasmania and *P. piscicida* strains PPSB25 and PPSB27 from ballast water from Indonesia (Table I). Their identifications were confirmed by SEM and DNA sequence analyses (Park *et al.*, 2007a). Burkholder *et al.* (2001a) claimed that toxicity can be lost in culture over time as an artifact of culture conditions. Thus, bioassays in this study were conducted using the *C. brodyi* and *P. piscicida* cultures within 1 year of isolation. The heterotrophic cultures were maintained in 35 psu f/2 medium at 20°C in the dark, and *Rhodomonas salina* (CS-24) was fed in 2–3 days intervals as a food source. *Rhodomonas salina* was cultured in 35 psu f/2 medium at 20°C under a 12:12 h light:dark cycle, of 100 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ light. Prior to larval bivalve

Table I: *Pfiesteria piscicida* and *C. brodyi* cultures used in this study

Culture code	Date of isolation	Locality	Identification
PPSB25	3 May	Surabaya, Indonesia, Ballast water	<i>Pfiesteria piscicida</i>
PPSB27	3 May	Surabaya, Indonesia, Ballast water	<i>Pfiesteria piscicida</i>
CBWA12	3 February	Brunswick River, Western Australia	<i>Cryptoperidiniopsis brodyi</i>
CBSA4	3 April	Port Lincoln, South Australia	<i>Cryptoperidiniopsis brodyi</i>
CBHU1	3 February	Huon River, Tasmania, Australia	<i>Cryptoperidiniopsis brodyi</i>
CBDE10	3 March	Sandy Bay, Derwent River, Tasmania	<i>Cryptoperidiniopsis brodyi</i>
CBDE1	4 March	Sullivans Cove, Derwent River	<i>Cryptoperidiniopsis brodyi</i>

bioassays, *Artemia* bioassays were conducted with the seven heterotrophic cultures. No mortality was found in the bioassays. *Cryptoperidiniopsis brodyi* strains CBWA12, CBSA4, CBHU1 and CBDE1 are genetically (based on rDNA sequences) unique Australian cultures compared to other regional isolates such as the USA, and they were widely found in Australia (Park *et al.*, 2007a). In contrast the two *P. piscicida* strains were tightly clustered with USA and Europe isolates by phylogenetic analysis based on rDNA sequences (Park *et al.*, 2007a). Thus, one each of the *C. brodyi* and *P. piscicida* strains (CBWA12 and PPSB25), representing the Australian genotype of *C. brodyi* and the ballast water isolates of *P. piscicida*, were chosen for further *Artemia* and larval bivalve bioassays.

Culture conditions of brine shrimp (*A. salina*)

Brine shrimp cysts (Aquafauna Bio-Marine, CA, USA) were placed in a 1 L beaker containing 700 mL of 35 psu sterile-filtered seawater with 0.2 μm membrane filter (PALL, MI, USA). The seawater was aerated from the bottom of the beaker using a standard aquarium aerator at 25°C to keep the cysts in suspension and allow for hatching. The cysts hatched between 24 and 48 h after being placed in the beaker. After hatching, small amounts of the green alga *Tetraselmis suecica* were fed as prey. *Tetraselmis suecica* was maintained in 35 psu f/2 medium at 20°C. The seawater in the beaker was exchanged by one third using fresh sterile-filtered seawater every alternate day. Nauplii hatched from cysts were allowed to grow to ~ 1 mm length for 2–3 days, and then used for the *Artemia* bioassays.

Bivalve (*C. gigas*) cultures

The oyster larvae (0.5–1, 2 and 4 mm length), which were pelagic and had not yet settled, were obtained from a local shellfish hatchery farm located in Frederick Henry Bay, Tasmania. The larvae were maintained in 35 psu seawater at 17°C with aeration. The water was exchanged with sterile-filtered seawater daily for minimization of microbial contaminants, and the larvae were fed with an algal diet (*T. suecica*). Prior to use of the larvae for bioassays, algal prey in their digestive system was allowed to clear for 2 days in the absence of any algal diet.

Design of larval bivalve bioassay

Preliminary bioassays were conducted to determine cell densities of negative and positive controls. Non-toxic *R. salina* was used for the negative control. Three oysters

of length 4 mm were inserted in each well of a 6 well tissue culture microplate (well diameter 35 mm) containing 8 mL of 35 psu filter-sterilized seawater. As a negative control, the oysters were tested with initial cell density of *R. salina* of 1000, 2000, 3000, 4000 and 5000 cells mL^{-1} for 12 days. The oysters with *R. salina* concentrations of ≤ 3000 cells mL^{-1} remained alive during the assay period. Based on this result, 3000 cells *R. salina* mL^{-1} was chosen for the cell density of the negative control. As a positive control, the dinoflagellate *Alexandrium catenella* (ACTRA02 isolated from Tasmania) producing PSP toxin was tested with 4 mm size oysters. A bioassay with initial cell density of 1000, 2000 and 3000 cells *A. catenella* mL^{-1} was conducted. All treatments with *A. catenella* showed 100% mortalities within 3 days. *Alexandrium catenella* of 1000 cells mL^{-1} was chosen for the positive control. A cell density of 2500 cells mL^{-1} of strains CBWA12 of *C. brodyi* and PPSB25 of *P. piscicida* for the bioassays was chosen at an intermediate level between those of the positive and negative controls. The oyster cultures inevitably bring microbial contaminants into the microplate, which can interfere with the bioassay. To minimize contaminants and maintain constant water quality, a semicontinuous batch culture was used for the bioassays. Eight milliliter of seawater per well was exchanged daily with 7 mL of sterile-filtered seawater containing 17 500 cells of *C. brodyi* (CBWA12) or *P. piscicida* (PPSB25), using disposable glass pipettes. The cell numbers were estimated using a hemocytometer (Blau Brand, Germany) under an Axioskop 2 Plus microscope (Zeiss, Gottingen, Germany) and the algal concentration in the microplates was adjusted daily to ~ 2500 cells mL^{-1} by exchanging the water with sterile-filtered seawater containing the known cell numbers. The microplates were also replaced with new plates at 6-day intervals. Oysters were transferred to wells with disposable plastic pipettes. The microplates containing oysters and algae were observed under an Axioskop 2 Plus microscope connected to AxioCam HR digital camera (Zeiss, Gottingen, Germany). Mortality of oysters was defined by the absence of ciliary movement for > 2 min, observed at 20 min intervals for 1 h. Each well of microplates contained 3, 5 and 12 oysters of 4 mm, 2 mm and 0.5–1 mm sizes, respectively. Bioassays with strains CBWA12 and PPSB25 were performed using oyster sizes of 0.5–1, 2 and 4 mm with the positive and negative controls, with each treatment consisting of 9 wells.

Artemia bioassays

Live cultures of *P. piscicida* and *C. brodyi* species were added directly into 6-well tissue culture microplates containing brine shrimp. Cell densities of the heterotrophic

algae, positive and negative controls were used at the same densities as those in the larval bivalve bioassay. To maintain constant cell densities during the 4-day assay period, seawater in the microplates was exchanged with fresh filter-sterilized seawater using the same method as described in the larval bivalve bioassay. The filtration and ingestion capacity of the brine shrimp during 1 day were also estimated by counting the number of *P. piscicida* or *C. brodyi* cells in the microplates at 0 and 24 h ($n = 6$). Twenty brine shrimp removed no more than 300 cells during 1 day; the algal cell density was assumed to have been approximately constant at 2460 to 2500 cells mL^{-1} . Twenty brine shrimps were placed in a well, and brine shrimp on the bottom of the plates with absence of movement for 5 min were regarded as dead. Each treatment consisting of six wells was monitored using light microscopy (LM) every day during the assay period.

Effect of *P. piscicida* and *C. brodyi* cell extracts on oyster larvae

The ability of *P. piscicida* and *C. brodyi* to cause oyster larvae mortality was also examined without direct contact between oysters and the algae. Seawater containing *P. piscicida* and *C. brodyi* was collected from the microplates used in the larval bivalve bioassays to induce oyster larvae mortality. The seawater with cells was sonicated for 1 min using a sonicator probe for rupturing the cells. The seawater was centrifuged for 15 min at $4000 \times g$ at room temperature, and the aqueous supernatants were retained and the cell pellets discarded. The aqueous solutions were then filter-sterilized using a syringe 0.2 μm filter membrane (low protein binding polycarbonate; PALL, MI, USA) to obtain bacteria-free medium. Aliquots of the aqueous solutions from each alga were freshly prepared daily and tested against the oysters. Four milliliter of the test solution was added to each well of microplates containing 4 mL of fresh seawater with 0.5–1 mm size larvae. Four milliliter of the supernatants in each well of the microplates was replaced daily with freshly prepared cell extracts. Each treatment consisted of six wells, and larval bivalve bioassays were performed for 9 days with the additions of small amounts of prey cells. Clonal cultures (10^4 cells mL^{-1}) of *P. piscicida* (PPSB25), *C. brodyi* (CBWA12) and *R. salina* were extracted as well, and those culture supernatants were used as controls.

Micropredations on oyster larvae and brine shrimp

Micropredation of *C. brodyi* on oyster larvae and brine shrimp was observed using LM connected to a digital

camera. A high cell density of 2×10^4 cells mL^{-1} of the algae was placed on a glass slide with 0.5 mm size oyster larvae or *Artemia*, and they were photographed.

Changes in cell numbers of zoospores and cysts with the presence of oyster larvae

Two treatments containing two plates (6 well tissue culture microplate, well diameter 35 mm) per treatment consisted of *C. brodyi* (CBWA12) cultures (cell density of 2×10^4 cells mL^{-1}) with additions of 12 larvae (0.5–1 mm size) per well and CBWA12 cultures combined in a ratio of 1:1 with *R. salina*. The cultures in the microplates were maintained as batch cultures at 20°C in the dark. Cell concentrations of both zoospores and cysts were measured daily for 5 days. Forty microliter of zoospores from each of 12 wells per treatment were fixed with 1% glutaraldehyde. Zoospore numbers were estimated using a hemocytometer (Blau Brand, Germany) under an Axioskop 2 Plus microscope (Zeiss, Gottingen, Germany). For measurements of the number of cysts, the microplate was placed under Axiovert 25 microscope (Zeiss, Gottingen, Germany) and cell numbers counted.

Data analysis

A repeated measures analysis of variance (ANOVA) was carried out using the computer program SAS (SAS Institute Inc, Cary, NC, USA). The ANOVA model was used to assess the changes in larval survivorship of the four cultures (*A. catenella*, *R. salina*, *C. brodyi* and *P. piscicida*) over time and the changes in cell numbers of zoospores and cysts over time.

RESULTS

Artemia bioassay

No mortality occurred in both treatments with *C. brodyi* and *P. piscicida*, respectively (Fig. 1D). All *Artemia* nauplii tested in the bioassays remained healthy during the bioassay period as well as in the negative controls. In the positive controls, mortality reached 95% after 24 h and 100% after 48 h. In the bioassays, active *Artemia* swam away from the zoospores, and hence no attraction of *C. brodyi* and *P. piscicida* to *Artemia* was observed. Figure 2B illustrates *C. brodyi* attached to live *Artemia* immobilized by heat shock. The zoospores exhibited less attraction to *Artemia*, compared to oyster larvae.

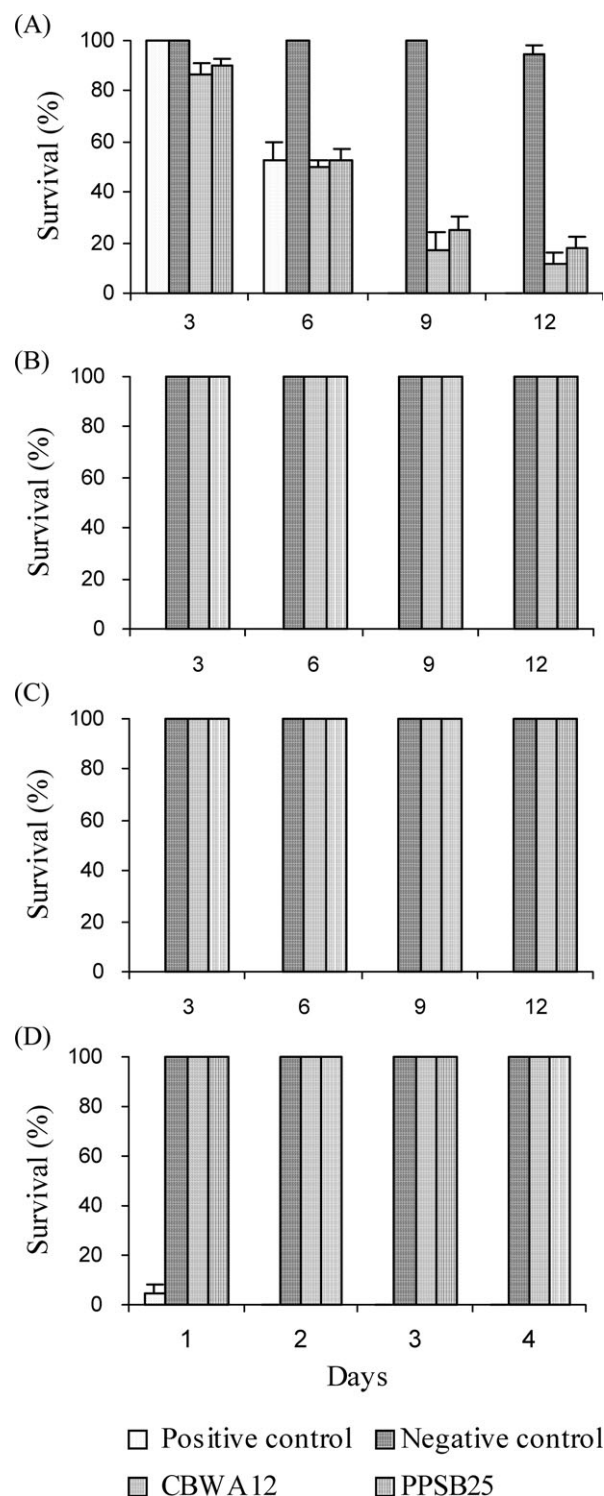


Fig. 1. Survivorship of oyster larvae (*C. gigas*) and *Artemia* nauplii in bioassays with *C. brodyi* (CBWA12) and *P. piscicida* (PPSB25), 2500 cells mL^{-1} of *C. brodyi* or *P. piscicida*, 1000 cells mL^{-1} of *A. catenella*, 3000 cells mL^{-1} of *R. salina* (A–C). (A) Survival of 0.5–1 mm oysters for 12 days. (B) Survival of 2 mm oysters for 12 days. (C) Survival of 4 mm oysters for 12 days. (D) Survival of *A. salina* nauplii in *Artemia* bioassays with *C. brodyi* and *P. piscicida* for 4 days. Positive control: PSP dinoflagellate *A. catenella*. Negative control: *R. salina*. Values are means \pm SE ($n = 3$). Statistical analysis of significance is shown in Table II.

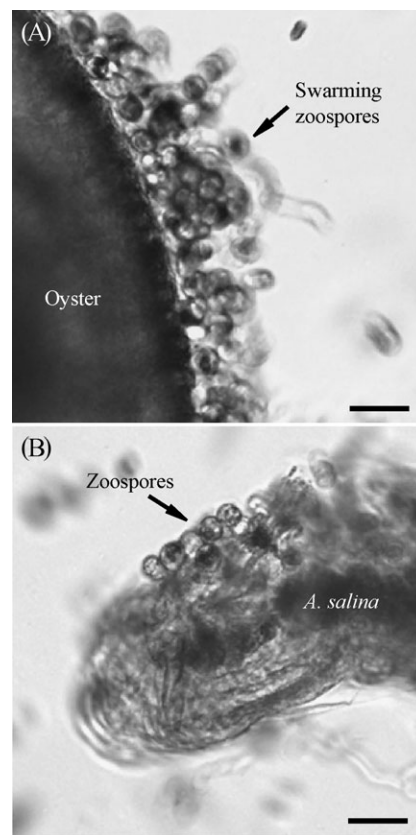


Fig. 2. *Cryptoperidiniopsis brodyi* (CBWA12). (A) Zoospores actively swarming around an oyster larva. (B) Zoospores attached to *A. salina*. *Artemia salina* was immobilized by heat shock to achieve this micrograph. Scale bars: 20 μm .

Larval bivalve bioassay

Significant effects of algal species ($P < 0.0001$) and time ($P < 0.0001$) on survival were found in the treatment with 0.5–1 mm size oysters (Table II; Fig. 1A). There was also a significant interaction between time and algal species on larval survival ($P < 0.0001$). In the 0.5–1.0 mm oyster treatment, oyster mortalities were 82% and 88% with *P. piscicida* and *C. brodyi*, respectively, after 12 days. There was no oyster death in the negative controls after 9 days, and limited mortality (5%) occurred on day 12. In the positive controls (0.5–1 mm size oysters) using *A. catenella*, the developing larval shells were mostly closed and ciliary movement was less active than negative controls. The first mortality in the positive controls appeared at day 6 and mortality reached 100% after day 9. During the bioassays, both *C. brodyi* and *P. piscicida* showed aggressive feeding behavior toward oyster larvae. Figure 2A illustrates strong attraction of *C. brodyi* toward oyster larvae. The zoospores swarmed around the oyster (0.5–1 mm size) and attached to the outer edge of the oyster shell within seconds.

Table II: Repeated-measures ANOVA assessing the changes in C. brodyi cell numbers with oyster larvae and survival of oyster with P. piscicida and C. brodyi

Source	Df	Ss	Mean square	F-value	Pr > F
Dependent variable: % oyster survival (Fig. 1a)					
Treatment	3	2.93×10^4	9.78×10^3	155.63	<0.0001
Error (treatment)	8	5.03×10^2	6.28×10	–	–
Time	3	3.06×10^4	1.02×10^4	280.28	<0.0001
Time × treatment	9	1.06×10^4	1.18×10^3	32.47	<0.0001
Error (time)	24	8.76×10^2	3.65×10	–	–
Dependent variable: zoospores mL ⁻¹ (Fig. 4a)					
Treatment	1	9.06×10^7	9.06×10^7	45.04	0.0026
Error (treatment)	4	8.04×10^6	2.01×10^6	–	–
Time	4	4.25×10^8	1.06×10^8	192.44	<0.0001
Time × treatment	4	4.07×10^7	1.01×10^7	18.43	<0.0001
Error (time)	16	8.83×10^6	5.52×10^5	–	–
Dependent variable: cysts mL ⁻¹ (Fig. 4b)					
Treatment	1	1.39×10^8	1.39×10^8	59.97	0.0015
Error (treatment)	4	9.30×10^6	2.32×10^6	–	–
Time	4	2.53×10^7	6.32×10^6	2.30	0.1032
Time × treatment	4	2.20×10^7	5.50×10^6	2.00	0.1426
Error (time)	16	4.39×10^7	2.74×10^6	–	–

Some zoospores had penetrated the inside of the oysters and showed attacking behavior, but the oysters survived predation by *C. brodyi* for 2 days, by creating turbulence using movement of the outer shell and cilia. At day 3, the zoospores began to consume the soft tissues including gill and mantle of weakened oysters. After feeding of the oysters by myzocytosis, the zoospores detached and swam away from the empty shells. However, a few oysters (12% of total) tolerated the attacking behavior of *C. brodyi* until the end of the bioassay (12 days) (Fig. 1A). Figure 3 illustrates myzocytosis of zoospores toward a small piece of oyster tissue. After 20 min (Fig. 3B), most of the soft tissues were consumed by the zoospores. When bioassays were conducted with aqueous cell extracts of *C. brodyi* or *P. piscicida*, no oyster deaths occurred in both treatments as well as controls.

In the treatments with oysters of 2 and 4 mm size, all oysters remained healthy, as indicated by active movement of their cilia (Fig. 1B and C). The oysters effectively avoided feeding by the heterotrophs through active and consistent movements of their outer shells, gill and extended vela. No mortality occurred in negative controls, while positive controls using *A. catenella* showed 100% mortality (0% survival) by day 3 (Fig. 1B and C).

Interaction between *C. brodyi* and oyster larvae

Cryptoperidiniopsis brodyi was maintained with oyster larvae, and the changes in cell numbers of zoospores

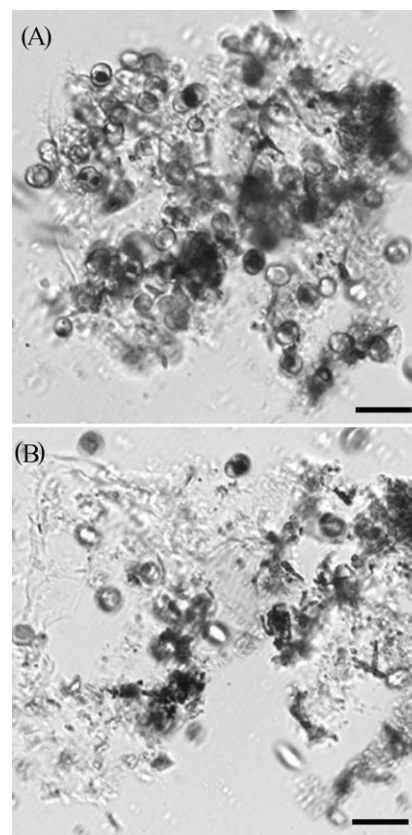


Fig. 3. Micropredation by *C. brodyi* (CBWA12). **(A)** Zoospores attached to and feeding on soft tissues of oyster. **(B)** After 20 min, most soft tissues were consumed by zoospores. Scale bars: 20 μ m.

and cysts were compared with those of treatments without oyster larvae. There were significant effects of time ($P < 0.0001$) and treatment ($P = 0.0026$) on zoospore numbers (Fig. 4A). There was also a significant interaction between time and treatment on zoospore numbers ($P < 0.0001$). In the treatment without oysters, zoospore numbers of *C. brodyi* declined rapidly during day 1 because most zoospores settled down on the bottom of the microplate and turned into temporary and division cysts. Subsequently, zoospore numbers gradually decreased by day 3–4, and then persisted on day 5. Higher zoospore numbers were evident in the presence of oysters than those in treatments without oysters. Even if the zoospore numbers were higher in the presence of oysters than without the presence of oysters, the numbers of zoospores were decreasing rapidly during the experiment in the oyster treatment. The treatment with oysters appeared to result in more active zoospores and swimming cells containing prey (mean zoospore numbers were 2.5-fold higher after 5 days). Zoospore numbers remained stationary after day 4.

With regard to cyst numbers produced during the experiment, there was a significant effect of treatment

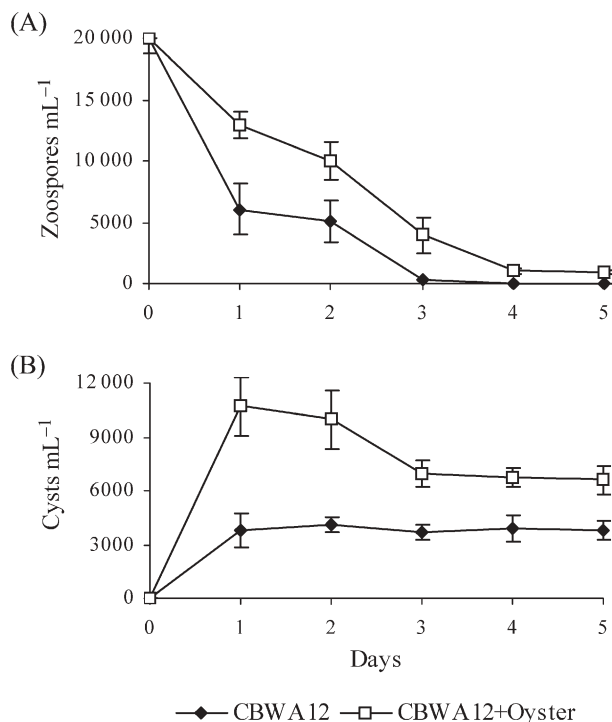


Fig. 4. *Cryptoperidiniopsis brodyi* (CBWA12). **(A)** Comparison of changes in zoospore numbers with and without oyster larvae (*C. gigas*) over 5 days. **(B)** Changes in cyst numbers during the bioassay experiment. Values are means \pm SE ($n = 12$). Statistical analysis of significance is shown in Table II.

($P = 0.0015$) on cyst numbers (Fig. 4B). At the same time, there were no significant effects of time ($P = 0.1032$) and interaction between time and treatment ($P = 0.1426$) on cyst numbers. Actively swarming zoospores clustered around the oysters and transformed to temporary cysts within seconds (Fig. 5). The treatment with oysters increased the abundance of cysts by day 1, with a decreased abundance by day 3, and then

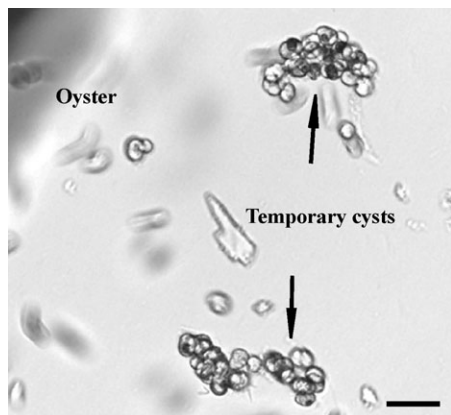


Fig. 5. *Cryptoperidiniopsis brodyi* (CBWA12). Temporary cysts produced from swarming zoospores around an oyster larva. Scale bars: 20 μ m.

stationary numbers afterwards (mean of 8186 cysts after 5 days). In contrast, the treatment without oysters showed smaller number of cysts and numbers remained steady after day 1 (mean of 3873 cysts after 5 days).

DISCUSSION

Predation on oyster larvae

Pfiesteria and *Pfiesteria*-like species possess feeding structures including a food vacuole and a peduncle, which enable them to consume various preys (Steidinger *et al.*, 1996, 2006). In the present study, aggressive feeding behavior by *C. brodyi* and *P. piscicida* was illustrated only toward *C. gigas* below 1 mm in size. A similar feeding response of *P. piscicida* has been reported previously toward planktonic bay scallop larvae (0.6 mm length), *Argopecten irradians* (Springer *et al.*, 2002). Myzocytosis of *Pfiesteria* species as observed by transmission electron microscopy has revealed that the distal end of an extended zoospore peduncle attached to epidermal tissue ingests organelles into a food vacuole within the zoospore epicone (Vogelbein *et al.*, 2002). Myzocytosis of *P. piscicida* and *C. brodyi* against oyster larvae indicates that these species can play a role as natural predators of shellfish in the marine environment. A previous study suggested that *Pfiesteria* spp. blooms of over 10^3 cells mL⁻¹ could adversely affect larval shellfish in natural waters (Shumway *et al.*, 2006). However, *C. brodyi* is usually present at a very low cell abundances (below 112 cells L⁻¹ in 2004–06) in Tasmanian waters, and cell densities never reached as high a level as used in the larval bivalve bioassays (Park *et al.*, 2007b). *Pfiesteria piscicida* was only detected once at 60 cells L⁻¹ during an 18 month field survey in Tasmanian waters by quantitative PCR based assay (Park *et al.*, 2007b). *Pfiesteria* species are known to feed on diverse prey sources, including bacteria, plankton, protozoan ciliates and rotifers (Burkholder and Glasgow, 1995). Prey selection of heterotrophic dinoflagellates may be affected by the size, shape and chemical cues of prey (Jeong and Latz, 1994; Strom and Buskey, 1993; Jeong, 1999; Martel, 2006). In the marine environment, various food sources can exist for those predators. The background food can satiate predators, and thereby reduce encounter between predators and bivalves (Johnson and Shanks, 1997; Johnson and Shanks, 2003). Unlike in small bioassay containers, turbulence in natural waters may also decrease encounters (Lewis and Pedley, 2001) and hence predation of *C. brodyi* and *Pfiesteria* species may have negligible impacts on early life stages of bivalves in local Tasmanian waters.

Larval bivalve and brine shrimp bioassays

The lack of mortality to oyster or *Artemia* in aqueous cell extract assays in the present study is in contrast to shellfish mortalities observed in bioassays where *P. piscicida* were prevented from direct contact with the shellfish (Springer *et al.*, 2002; Shumway *et al.*, 2006). Brine shrimp assays have also sometimes demonstrated that *P. piscicida* extracts can be lethal to *Artemia* (Moeller *et al.*, 2001). From this, it was suggested that *Pfiesteria* is able to produce toxins when maintained on fish prey but not when cultured with algal prey, with non-inducible strains never being capable of producing a toxin (Burkholder and Glasgow, 1997a, b; Burkholder *et al.*, 2001a, b, c, 2005). However, the aquarium fish bioassay system used in some of these studies includes microorganisms such as bacteria, fungi, protozoa and amoebae (Drgon *et al.*, 2005). Fish pathologies in a laboratory situation are commonly associated with fungal, bacterial and protozoan infections (Blazer *et al.*, 1999, 2000; Noga, 2000; Kiryu *et al.*, 2003; Drgon *et al.*, 2005; Stine *et al.*, 2005). Contaminants introduced into *Pfiesteria* cultures by the presence of fish complicate the identification of the causative agent responsible for toxicity (Coats, 2002). For this reason, *Pfiesteria* cultures have more recently been separated from direct contact with fish and isolated from contaminants introduced by fish to examine if bioactive substances or toxins from *Pfiesteria* cause mortalities of fish (Vogelbein *et al.*, 2002; Berry *et al.*, 2002; Lovko *et al.*, 2003). In some experiments, cell-free filtrate was unable to induce fish mortality but fish kills occurred when fish were physically attacked by feeding behavior of *Pfiesteria*. In studies by Moeller *et al.* (2001), Springer *et al.* (2002) and Shumway *et al.* (2006), *Pfiesteria* cultures were maintained with live fish prior to larval bivalve or brine shrimp assays. Subsequently, culture medium containing *Pfiesteria* was collected from the tanks in which fish kills occurred, and tested in bioassays. Accordingly, it could not be conclusively established whether mortalities of shellfish and brine shrimp were caused by toxins from *Pfiesteria* or tank contaminants.

In the present study, micropredation by *C. brodyi* induced mortalities of planktonic oyster larvae. Marshall *et al.* (2000) previously reported that *Cryptoperidiniopsis* species did not initiate fish deaths in bioassays. In studies by Burkholder, 3 out of 15 *Cryptoperidiniopsis* clones caused >50% fish death in bioassays, but the mortalities appeared to result from physical attack only (Burkholder *et al.*, 2001a). Low larval fish mortalities by physical attack were also reported in fish microassays with *Cryptoperidiniopsis* species (Burkholder *et al.*, 2005). It appears that *C. brodyi* is a heterotrophic dinoflagellate

exhibiting feeding behavior toward preys such as algae, shellfish and fish, but their predatory behavior varies from strain to strain and with the precise conditions of bioassay.

The present study showed that the initial mortality of 0.5–1 mm size oyster larvae occurred at a magnitude of 10–13% on day 3 at 2500 cells mL⁻¹ of *P. piscicida* or *C. brodyi*. In comparison, however, Springer *et al.* (Springer *et al.*, 2002) reported that oyster larvae (mean length 0.6 mm) developed a noticeable valve gape at 5000 cells mL⁻¹ of *P. piscicida* and their first mortalities appeared after 15 min. The different times of the oyster death may be due to different *P. piscicida* density used in the bioassays and this may indicate that oyster larvae mortality may be associated with increased cell densities of *P. piscicida*. For example, a positive correlation between *P. piscicida* density and fish larvae mortality has been reported when fish larvae were exposed to various *P. piscicida* densities for 16 h (Drgon *et al.*, 2005). Mortalities of fish larvae were also greater and occurred more rapidly with increasing *P. shumwayae* density for 96 h (Lovko *et al.*, 2003). Admittedly, it would have been preferable to have repeated the larval bivalve bioassay with a series of different *P. piscicida* or *C. brodyi* cell densities.

In the positive controls with *A. catenella*, 100% mortality of 2 or 4 mm size oysters occurred within 3 days while 0.5–1 mm size oysters took 6 days for the first mortality to appear and there was 100% mortality at day 9. It is not clear why 0.5–1 mm size oysters took longer for the first mortality to appear than the other oyster sizes, even though the bioassays were conducted at the same concentration of *A. catenella* (1000 cells mL⁻¹). One possibility is that the susceptibility of the oyster toward algal toxins may vary for individual oyster larvae. Feeding oysters with toxigenic dinoflagellates such as *Alexandrium* spp. induced significant inhibition of shell valve activity and feeding rate (Bricelj *et al.*, 1991; Lassus *et al.*, 1999). It has been reported that the oyster *C. gigas* fed with *A. minutum* Halim showed considerable inter-individual variability of feeding activity and toxin accumulation rates (Bougrier *et al.*, 2003).

Growth of the dinoflagellate in the presence of oyster larvae

Zoospores of *C. brodyi* and *P. piscicida* exhibited a strong attraction and feeding behavior toward oyster larvae. When maintained without algal prey, a higher abundance of zoospores and cysts of *C. brodyi* was evident in treatments with oyster larvae than those without oyster larvae. Chemosensory response is defined as the

attraction or repulsion of organisms in concentration gradients of chemical substances (Bean and Yussen, 1979). It has been suggested that chemosensory signals given off by different prey types may play a role in feeding selectivity of heterotrophic dinoflagellates (Hauser *et al.*, 1975; Buskey, 1997). For example, the phagotrophic dinoflagellate *Gymnodinium fungiforme* (considered to be closely related or even identical to *Cryptoperidiniopsis*) is attracted to a variety of amino acids and other organic compounds which are common in invertebrates (Spero, 1985). Previous studies have shown that *Pfiesteria* and *Cryptoperidiniopsis* are positively chemotactic toward mucus and excreta from fish (Cancellieri *et al.*, 2001). The chemosensory response toward fish materials has been claimed to be not a simple nutritional response to organic molecules but a more specific stimulation by a substance in fish mucus and excreta (Cancellieri *et al.*, 2001). It has also been shown that interaction between bacteria and dinoflagellates could affect both the growth and life cycle of the dinoflagellates. For example, cell surface hydrophobicity in bacteria could promote predation of heterotrophic marine nanoflagellates (Monger *et al.*, 1999). A species in the marine α -bacteria group is known to be beneficial to growth and physiology of *P. piscicida* and related dinoflagellates (Alavi *et al.*, 2001). Certain bacteria similar to *Roseobacter* sp. increased the consumption rate of algal prey and enhanced the growth of *P. piscicida* (Alavi, 2004). The role of bacteria in shellfish/*C. brodyi* interactions is not yet known, but bacteria co-existing with shellfish larvae or *C. brodyi* could be one of the factors to promote predation toward shellfish larvae and to be advantageous to the growth of the dinoflagellate.

In conclusion, this study demonstrated that aqueous cell extracts of *C. brodyi* or *P. piscicida* did not cause oyster death while their feeding responses could induce mortalities of planktonic oyster larvae. *Cryptoperidiniopsis brodyi* was also attracted to oyster larvae and its growth decreased less with the presence of oysters than without oysters. However, considering the very low abundances of these dinoflagellates in Tasmanian estuaries, *C. brodyi* and other related dinoflagellates are expected to usually have little or no adverse impact on planktonic bivalve larvae in nature.

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